UNIVERSITI PUTRA MALAYSIA

MOLECULAR AND BIOLOGICAL CHARACTERIZATION OF TWO VERY VIRULENT INFECTIOUS BURSAL DISEASE VIRUS ISOLATES, UPM94/273 AND UPM97/61

KONG LIH LING

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By

KONG LIH LING

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

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Dedicated with love and gratitude to:

My dearest parents, fiance, family and five lovely nieces

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chairman: Associate Professor Dr. Abdul Rahman Omar, Ph.D.

Faculty: Veterinary Medicine

An atypical very virulent (vv) strain (UPM94/273) and typical vv strain (UPM97/61) of infectious bursal disease virus (IBDV) isolated in Malaysia, were characterized both *in vivo* and at the molecular level. Comparison of the deduced amino acid sequences with other serotype 1 and 2 sequences revealed 16 amino acid residues, which were conserved only in the vvIBDV. Among the 16 unique amino acid differences, 8 were in VP1 (146 Asp, 147 Asn, 242 Glu, 390 Met, 393 Asp, 562 Pro, 687 Pro and 695 Arg), 3 were in VP2 (222 Ala, 256 Ile and 294 Ile), 2 were in VP3 (990 Val and 1005 Ala) and 3 were in VP4 (685 Asn, 715 Ser and 751 Asp). The importance of these unique amino acid residues is not known but they could affect the virulence of vvIBDV. The UPM94/273 also demonstrated 6 unique amino acid residues at segment

A at positions Ser254, Glu270, Lys588, Ser745, Phe838 and Lys863 and 8 unique amino acid residues at segment B at positions Ala92, Ser100, Val208, Asp253, Asp560, Asn565, Gly750 and Gly876. In addition, these amino acid substitutions have not been reported before in vvIBDV and were found only on variant, classical and/or serotype 2 strains. However, the VP5 region of both vvIBDV strains was conserved. The UPM97/61demonstrated 7 unique amino acid substitutions at segment A and 4 unique amino acid substitutions at segment B. However, none of the amino acids changes have been reported elsewhere in other IBDV strains. Although the actual functions of the amino acid substitutions are not know, the unusual amino acid substitutions at segment A and/or B of both isolates may be important in virus virulence. Alignments of the nucleic acid and amino acid sequences of segment A and B followed by distance analysis allowed the generation of phylogenetic trees. Phylogenetic analysis based on segment A and B revealed that all the vvIBDV strains including UPM94/273 isolate can be clustered in a group that is distinct from classical, variant, attenuated and serotype 2 strains. However, the tree branching patterns were quite different between segment A and segment B. in addition, the vvIBDV strains showed several conserved amino acid substitutions at segment B as found in the Australian 002-73 and serotype 2 strains. These findings indicate that probably a genetic reassortment may have play an important role in the emergence of vvIBDV. Flow cytometry and real time PCR assays, indicated that chickens infected with UPM97/61 induced higher

percentages of apoptotic cells but lower level of viral load whereas UPM94/273 induced lower percentages of apoptotic cells but higher level of viral load, suggesting a negative correlation between viral load and apoptosis. These results indicated that UPM97/61 was more virulent than UPM94/273.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENCIRIAN MOLEKUL DAN BIOLOGIK ISOLAT-ISOLAT UPM94/273 DAN UPM97/61 VIRUS PENYAKIT BURSA BERJANGKIT

Oleh

KONG LIH LING

Februari 2003

Pengerusi: **Profesor Madya Dr. Abdul Rahman Omar, Ph.D.**

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Virus penyakit bursa berjangkit amat virulen (vvIDBV) yang tidak khusus (UPM94/273) dan yang khusus (UPM97/61) diperolehi dari Malaysia, telah dicirikan *dalam vivo* dan berdasarkan molekul. Perbandingan jujukan asid amino dengan jujukan serotip 1 dan 2 yang lain telah menunjukkan 16 asid amino residu yang cuma terdapat di vvIBDV. Perbezaan antara 16 asid amino yang unik ini, 8 terdapat di VP1 (146 Asp, 147 Asn, 242 Glu, 390 Met, 393 Asp, 562 Pro, 687 Pro dan 695 Arg), 3 terdapat di VP2 (222 Ala, 256 Ile dan 294 Ile), 2 terdapat di VP3 (990 Val dan 1005 Ala) dan 3 terdapat di VP4 (685 Asn, 715 Ser dan 751 Asp). Kepentingan residu-residu asid amino ini adalah tidak diketahui tetapi ia boleh mempengaruhi virulen vvIBDV. UPM94/273 juga menunjukkan 6 asid amino yang unik pada kedudukan Ser254, Glu270,

Lys588, Ser745, Phe838 dan Lys863 mana setiap 2 berada di VP2, VP3 dan VP4. Tambahan pula, penggantian asid amino pada kedudukan Gly254Ser, Ala270Glu, Glu588Lys dan Asp745Ser cuma terdapat di varian, klasik dan/atau serotip 2. Walaubagaimanapun, bahagian VP5 untuk kedua-dua vvIBDV adalah kekal. UPM97/61 menunjukkan 7 penggantian asid amino yang unik di segmen A dan 4 penggantian asid amino yang unik di segmen B. Tetapi, perubahan asid amino ini tidak pernah dilaporkan pada strain-strain IBDV yang lain. Walaupun fungsi sebenar penggantian asid amino adalah tidak diketahui, tetapi penggantian asid amino yang luar biasa ini pada segmen A dan/atau B untuk kedua-dua isolat mungkin penting dalam virulen virus. Susunan untuk jujukan asid nukleik dan asid amino untuk segmen A dan B diikuti oleh analisis jarak membenarkan pembentukan pokok filogenetik. Analisis filogenetik berdasarkan segmen A dan B menunjukkan semua strain vvIBDV termasuk isolat UPM94/273 IBDV boleh dikumpulkan dalam satu kumpulan di mana adalah berbeza daripada strain-strain klasik, varian, akenuat dan serotip 2. Walaupun begitu, corak-corak untuk cabangan pokok adalah berlainan antara segmen A dan segmen B. Tambahan pula, strain-strain vvIBDV menunjukkan beberapa penggantian asid amino yang kekal dalam segmen B seperti yang dijumpai dalam strain-strain Australian 002-73 dan serotip 2. Penemuan ini menyatakan bahawa kemungkinan penyusunan semula genetic yang mungkin memainkan peranan yang penting dalam kemunculan vvIBDV. Cara-cara aliran sitometri dan PCR masa sebenar, menyatakan bahawa

ayam-ayam yang dijangkiti dengan UPM97/61 mengakibatkan peratusan sel-sel apoptosis yang lebih tinggi tetapi takat muatan virus yang lebih rendah manakala UPM94/273 mengakibatkan peratusan sel-sel apoptosis yang lebih rendah tetapi takat muatan virus yang lebih tinggi, mencadangkan satu perhubungan yang negatif wujud di antara muatan virus dan apoptosis. Keputusan ini menyatakan bahawa UPM97/61 adalah lebih virulen daripada UPM94/273.

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I certify that an Examination Committee met on 25th February 2003 to conduct the final examination of Kong Lih Ling on her Master of Science thesis entitled "Molecular and Biological Characterization of Two Very Virulent Infectiuos Bursal Disease Virus Isolates, UPM94/273 and UPM97/61" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I here declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

KONG LIH LING

Date: 2/5/2003

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LIST OF ABBREVIATIONS

AGPT	Agar gel precipitin test
BLAST	Basic local alignment search tool
bp	Basepair
BSA	Bovine serum albumin
CAM	Chorioallantoic membrane
cDNA	Complementary deoxyribonucleic acid
°C	Degree Celcius
CE	Chicken embryo
CMX-Ros	Chloromethyl-X-rosamine
CT	Threshold cycle
DEPC	Diethyl pyrocarbonate
DH_2O	Distilled water
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
ds	Double strand
DTT	Dithiothreitol
dUTP-FITC	dUTP flurorescein isothiocyanate
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
EID_{50}	Embryo infective dose fifty
ELISA	Enzyme linked immunosorbent assay
HVT	Herpes virus of turkey
IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
IFN	Interferon
IPNV	Infectious pancreatic necrosis virus
kb	Kilobase
kDa	Kilodalton
LB	Luria-Bertani
M	Molar
MC-540	Merocyanine-540
MDA	Maternally derived antibody
$MgSO_4$	Magnesium sulfate
ml	Millilitre
mM	Millimolar
µg	Microgram
NaCl	Sodium Chloride
NaI	Sodium iodide
NaOH	Sodium hydroxide
NCBI	National centre for biotechnology information
ng	Nanogram
NJ	Neigbour-joining
OD	Optical density
ORF	Open reading frame

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pi	Post infection
PI	Propidium iodide
pmol	Picamol
PTC	Peltier thermal cycler
QC-PCR	Quantitative competitive PCR
QGDPT	Quantitative gel diffusion precipitin test
RdRp	RNA dependent RNA polymerase
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT-PCR	Reverse-transcriptase PCR
RT	Room temperature
SDS	Sodium dodecyl sulfate
SPF	Specific pathogen free
SPSS	Statistical program for social science
TAE	Tris-acetate-EDTA
TCVN	Tissue culture virus neutralization
Tris	2-amino-2-(hydroxymethyl)-1, 3 propandiol
TUNEL	Terminal deoxynucleotidyl transferase mediated nick and labeling
UPGMA	Unweighted pair group method with arithemetic mean
UPM	Universiti Putra Malaysia
UV	Ultraviolet
VNF	Virus neutralizing factor
VP	Viral protein
vv	Very virulent
(w/v)	Weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Amino Acid	Single/Three Letter Amino Acid Code	
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic Acid	D	Asp
Glutamine	Q	Gln
Glutamic Acid	E	Glu
Glycine	G	Gly
Isoleucine	I	Ile
Leucine	L	Leu
Lycine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Valine	V	Val

CHAPTER I

INTRODUCTION

Infectious bursal disease (IBD) is an acute contagious viral disease of young chickens (Kibenge *et al.*, 1988; Lasher *et al.*, 1994). The etiological agent, IBD virus (IBDV), has a predilection for the cells of the bursa of Fabricius where the virus infects actively dividing and differentiating lymphocytes of the B-cell lineage (Burkhardt *et al.*, 1987). Thus, IBD is a fatal immunosuppressive disease causing heavy losses to the poultry industry (Eterradossi *et al.*, 1998).

The first outbreak of IBD was reported in commercial chicken flocks in Delaware, USA (Cosgrove, 1962). The IBDV strains, which were isolated during this outbreak, are now referred to as classical serotype I isolates. Later on, a second serotype – serotype II of IBDV was identified (McNulty and Saif, 1988). These isolates are apathogenic and are recovered mainly from turkeys (Ismail *et al.*, 1988). Based on antigenic variation and virulence, serotype I isolates can be divided into several groups : classical virulent, attenuated, antigenic variant and very virulent (vv) strains (Cao *et al.*, 1998). Since 1985, antigenic variants of serotype I IBDV isolates had been recovered from flocks with selection pressure of field vaccination against classical IBDV serotype I (Snyder, 1990). Although being antigenic variant, these isolates have only minor amino

acid changes and do not form any separate serotype. Nevertheless, these changes occur at the VP2 conformation-dependent antigenic epitopes that are responsible for stimulating virus neutralizing antibodies (Bayliss *et al.,* 1990). In 1991, IBDV isolates, which were able to break through levels of maternal antibodies that were normally protective, were reported in Europe (Chettle *et al*., 1989). These isolates, the so called very virulent IBDV (vvIBDV), cause more severe clinical signs during an outbreak with mortality approaching 100% in susceptible flocks, and are now found almost world-wide (VandenBerg, 2000).

The emergence of highly virulent strains of IBDV has complicated the immunization programs against the disease. Early vaccination may result in failure due to interference with the maternal antibody, whilst its delay may cause field virus infections. Therefore, it is important to characterize the antigenicity and the virulence of IBDV in both vaccine and field strains in the control of the disease. The effectiveness in the latter is also dependent on the diagnostic methods used. The disease can be diagnosed based on virus isolation, electron microscopy, immunofluorescence, virus neutralization, monoclonal antibody assays, and/or enzyme-linked immunosorbent assay (Lukert and Saif, 1991; Wu *et al*., 1992; Liu *et al*., 1994). However, these methods have one or more disadvantages such as being time consuming, labour intensive, expensive and of low sensitivity (Wu *et al*., 1992).

Recently, more sensitive and specific molecular methods have been used to diagnose and characterize IBDV infections (Jackwood and Nielsen, 1997; Moody *et al.*, 2000; Boot *et al.*, 2001). The reverse transcriptase polymerase chain (RT-PCR) has been widely used to detect IBDV (Tham *et al.*, 1995; Jackwood and Nielsen, 1997). RT-PCR followed by restriction fragment length polymorphism (RFLP) has also been used to detect and differentiate IBDV strains (Jackwood and Sommer, 1997; Hoque *et al.,* 2001). RT-PCR RFLP profiles of the amplified hypervariable region of the VP2 gene have been used to diagnose and identify molecular differences in the IBDV strains isolated in different parts of the world (Jackwood and Sommer, 1999; Hoque *et al.,* 2001). In these studies, it was found that all vvIBDV isolates have a conserved *Ssp*1 and *Taq*1 sites at the hypervariable region of the VP2 gene. A study has also been carried out to develop a PCR method for the detection of IBDV based on colorimetric technique (Phong, 2002).

Generally the severity of IBDV infections has been assessed in terms of mortality or the degree of bursal damage, and it has been difficult to assess viral load because virulent strains of IBDV do not replicate in tissue culture (Moody *et al.*, 2000). A quantitative competitive PCR (QC-PCR) assay has been developed to monitor IBDV RNA extracted from infected bursae (Wu *et al.*, 1997). However, this protocol is labour intensive and the technique has limitations (Souaze *et al.*, 1996). Difficulties in using the RT-PCR technique for quantification